Turkish Journal of Agriculture - Food Science and Technology, 12(s2): 2266-2271, 2024
DOI: https://doi.org/10.24925/turjaf.v12is2.2266-2271.7002

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

In-vitro Antimicrobial Activity of ZnO Nanoparticles Produced by Hydrothermal Method Against Some Foodborne Pathogens

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ARTICLE INFO

Research Article

Received : 13.07.2024
Accepted : 26.08.2024

Keywords:
ZnO
Nanoparticle
Antimicrobial activity
Foodborne pathogens
Characterization

ABSTRACT

Zinc oxide nanoparticles (ZnO-NPs) are synthesized via a multitude of techniques, resulting in nanoparticles of varying sizes and morphologies that directly influence their antimicrobial efficacy. The objective of this study is to ascertain the particle size and morphology of ZnO-NPs synthesised via the hydrothermal method and to evaluate their *in vitro* antibacterial effects against *Escherichia coli* O157, *Salmonella* Typhimurium, and *Listeria monocytogenes*, which are important foodborne pathogens. The ZnO-NPs were examined using a scanning electron microscope (SEM). Furthermore, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the diameter of inhibition zones were measured against these pathogenic bacteria. The SEM images revealed that the ZnO-NPs exhibited a uniform distribution, with particle sizes ranging between 23 and 25 nm. The MIC and MBC values against the tested strains were found to range from 20.83 to 41.67 µg/mL and between 66.67- 83.33 µg/mL, respectively. In addition, the diameter of inhibition zones were ranged from 15.16 to 16.96 mm. The findings of the study demonstrated that ZnO-NPs s synthesized via the hydrothermal method exhibited antibacterial effects against both Gram-positive and Gram-negative bacteria. In conclusion, the use of ZnO-NPs can facilitate the improvement of the microbiological quality of foods by the inhibition of foodborne patogens.

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Introduction

Nanotechnology represents a promising domain of technological advancement with a considerable influence on human well-being and social conditions (He et al., 2019). The advent of nanotechnology has given rise to a multitude of pioneering developments across a spectrum of areas, including pharmacy, medical sciences, agriculture, food preservation, textiles, and environmental and energy resources (Adeyemi & Fowel, 2023; AL-Tamimi, 2021). Furthermore, the fields of nanoscience and nanotechnology have assumed to play a prominent role in food science research, largely due to their profound impact on the overall preservation and safety of food products (Leta et al., 2024). For instance, the incorporation of nanomaterials into food packaging has apotential to facilitate a profound transformation in the manner by which food is stored, transported, and consumed. This is an emerging area that provides solutions to improve food safety, prolonging shelf life, and mitigate environmental impact (Gupta et al., 2024). This technology focuses on developing and utilizing new edible materials at the nanoscale, collectively known as nanoparticles. Nanoparticles, defined as materials with dimensions ranging from 1 to 100 nm, possess distinctive chemical and physical properties that render them suitable for a wide range of applications (Leta et al., 2024). Specifically, nanoparticles are extensively used in the production of food packaging materials due to their antimicrobial properties. These properties allow them to effectively inhibit the growth of different microorganisms, including bacteria, yeasts, molds, and specific pathogens such as *Salmonella* Typhimurium, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Candida albicans*, and *Lactobacillus plantarum* (Gur et al., 2022; Leta et al., 2024; Rout & Pradhan, 2024). It is also well documented that NPs with antimicrobial and antioxidant properties may assist in maintaining the aroma and flavor of foodstuffs by inhibiting or retarding the microbiological and oxidative deterioration of foods. (Berekaa, 2015).

NPs are generally divided into two main categories based on their fundamental properties as follows: organic and inorganic NPs. Inorganic NPs, including CuO-, ZnO-, TiO_2-, Ag-, and Au-NPs, have been extensively used in food technology, particularly in edible films. Incorporating these materials in packaging has a potential to improve the physicochemical and functional properties of foods (Gupta et al., 2024; Kevenk & Aras, 2022).

Metal and metal oxide NPs, including silver, aluminum oxide, iron oxide, silica oxide, titanium oxide, zinc oxide, and copper oxide, have garnered attention in recent years due to their exceptional physicochemical properties, including stability and antimicrobial effects (Gur et al., 2022). In this context, ZnO is a highly versatile material with a range of physicochemical properties, including mechanical, electrical, optical, magnetic, catalytic, and strong piezoelectric and pyroelectric characteristics. ZnO has been employed in a number of different applications, including the manufacture of electronic devices, the packaging of food products, the treatment of wastewater, and the development of biomedical technologies including drug delivery systems, as well as anticancer, antibacterial, and antidiabetic activities (Jamdagni et al., 2018; Phatak et al., 2024).

ZnO nanoparticles are classified as inorganic NPs and have gained a significant attention from researchers due to their outstanding properties, which include low toxicity, physicochemical characteristics, and environmental friendly behavior. In recent years, ZnO-NPs have been assigned as Generally Recognized as Safe (GRAS) products under 21CFR182.8991 by the United States Food and Drug Administration (FDA) (Rout & Pradhan, 2024).

The nanostructure of ZnO has been found to demonstrate antibacterial effects through the generation of Zn^{2+} ions within microbial environments. The release of these ions disrupts amino acid metabolism and enzyme systems in microorganisms. The release of Zn^{2+} is primarily influenced by two main factors: (1) the physicochemical properties of the particles, including size, porosity, concentration, and morphology; (2) environmental conditions like pH, duration of exposure, and the presence of additives (Gupta et al., 2024). In this context, Dutta et al. (2012) reported that ZnO-NPs exhibited antibacterial properties by generating reactive oxygen species (ROS) in the culture media which induced the peroxidation of the lipid membrane of the microorganisms.

The synthesis of ZnO-NPs can be achieved through a number of different physical or chemical methods. A variety of techniques have been employed for the synthesis of ZnO-NPs, including ball milling, laser ablation, chemical and physical vapour deposition, ion implantation, electron beam evaporation, and thermal evaporation. Physical techniques like laser ablation yield ZnO with a consistent shape, while ball milling provides a cost-efficient method for producing nanocrystalline ZnO powder. Nevertheless, physical methods pose several challenges, such as requiring high pressure and temperature, involving high equipment costs, and presenting difficulties in controlling synthesis parameters. Chemical methods, such as solvothermal, hydrothermal, precipitation, sol-gel, sonochemical, polyol, and chemical reduction methods, have been developed for the synthesis of ZnO nanoparticles as well (Nawaz et al., 2024).

In line with the advancement of knowledge in the field of hygiene over the past decade, the significance of nanoparticles has grown on a global scale. NPs have begun to emerge in numerous contexts as a novel, efficacious, and supplementary instrument in the battle against zoonotic diseases, one of the most pressing public health concerns in the present era (Deshmukh et al., 2019). According to the World Health Organization (WHO), about 600 million cases of foodborne illness and 420,000 deaths are linked to foodborne pathogens each year. As indicated in the 2022 zoonoses report published by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), *Salmonella* spp., Shiga toxin-producing *E. coli*, and *L. monocytogenes* are among the five most prevalent zoonotic agents affect humans (EFSA & ECDC, 2022). It is therefore of the utmost importance to address foodborne illnesses caused by foodborne pathogens. (Leta et al.,2024).

Interest in NPs significantly increased only in the latter half of the 20th century, primarily due to the prior absence of technologies capable of accurately evaluating their antimicrobial and physicochemical properties. As a consequence of scientific developments, there has been a considerable increase in research activity over the past two decades. However, as previously stated, the NPs synthesized via different approaches may have undergone alterations in their bioactive properties, including the antimicrobial effect. Therefore, there is still need to evaluate the antimicrobial activity of the NPs produced by different methodologies. In this regard, the study aims to identify the particle size and morphology ZnO-NPs by produced hydrothermal methodology and investigation of its *in-vitro* antimicrobial effect against major foodborne pathogenic strains, including *L. monocytogenes* (ATCC 13932), *E. coli* O157:H7 (ATCC 35150) and S. *Typhimurium* (ATCC 14028).

Materials and Methods

Synthesis of ZnO-NPs

NPs were produced via the hydrothermal methodology. Briefly, 4 g of zinc acetate were dissolved in 60 mL of double-distilled water. Then, 2 M NaOH was added to the solution until the pH reached 13.0. The resulting mixture was then homogenised for 15 min via ultrasonication. Subsequently, the mixture was transferred to a teflon vessel within the Fytronix Nanomaterial Production Device (Fytronix Corp, Türkiye) where it was heated to 180 °C for 8 h. Following this, the resulting mixture was subjected to filtration and washing three times with double-distilled water. It was then placed in a petri plates and dried for 12 h at 80°C in a vacuum oven (Erol et al., 2022).

Scanning Electron Microscopy

The ZnO-NPs was characterised by taking SEM images. The analyses were carried out at the Yuzuncu Yil University Scientific Application and Research Centre (Van/Türkiye). The ZnO-NPs were examined in terms of shapes and size distributions (SEM; ZEISS Sigma 300, 10 kV, 100.00 K× magnification). The images were used to gather detailed information about the particle sizes and distributions of the NPs.

In Vitro Antibacterial Activity

The *in-vitro* antimicrobial activity tests were conducted on the day of the synthesis of ZnO-NPs against three pathogens: *L. monocytogenes* (ATCC 13932), *E. coli* O157:H7 (ATCC 35150), and *S.* Typhimurium (ATCC 14028). For this purpose, measuring the diamater of the inhibition zones, MIC and MBC tests were performed. In the inhibition zones assay, the disc diffusion method was employed, and the diameters of the zones were measured. In brief, 100 µL of inoculum of the tested pathogens with a concentration of 6.0 $\log_{10}$ CFU/mL were spread on Mueller-Hinton agar. After a five-minute incubation period at ambient temperature to allow for microbial adherence, a solution of ZnO-NPs at a concentration of 400 µg/mL (20 µL) was added to the plates and the plates incubated at 37 °C for 24 h. Subsequently, the diamaters of inhibition zones were measured with digital callipers (Moradi et al., 2019). MIC values were determined through the broth dilution method. A solution of ZnO-NPs at a concentration of 200 µg/mL was prepared in Tryptic Soy Broth, and two-fold serial dilutions were subsequently prepared with the same broth. A 100 µL of pathogenic bacteria inoculum at a concentration of 6.0 $\log_{10}$ CFU/mL was added to the tubes. Following incubation period (37 °C for 24 h), the MIC was identified as the lowest concentration of ZnO-NPs with no visible bacterial growth. The MBC value was then determined following the MIC assay. Briefly, 10-µL from each tube containing concentrations of MIC to MIC ×8 were spread onto MHA plates. After 24 hours of incubation at 37 °C, the minimum concentration that completely inhibited the tested strains was determined as the MBC value (CLSI, 2017).

Statistical Analyses

In the present study, data were obtained from three independent replicates. The statistical analyses were conducted using the SPSS software, version 21.0. All data sets were presented as mean ± standard deviation, and a p-value of 0.05 was considered statistically significant.

Results and discussion

As shown in Figure 1, the SEM image revealed that the particle size ranges between 23 and 25 nm. The particle sizes of the synthesized NPs provided an evidence that nanoparticles were successfully synthesized via the hydrothermal method. Consistently, Donmez and Keyvan (2023) found that the particle size of the ZnO-NPs produced by grape seed extract via green synthesis process was found to be 15.86 nm. . In another study conducted by Alizadeh-Sani et al. (2020), the mean diameter of ZnO-NPs was found to be approximately 20 nm. The ZnO-NPs particle sizes obtained by the researchers are comparable to those observed in our current study. In addition, the results indicate that the using of different techniques exerts a profound influence on the characteristics of the NPs. This may lead to alterations in their structural and physicochemical properties, which could potentially change their antimicrobial efficacy due to increased accessibility to microorganisms.

It is well-established that one of the principal antimicrobial mechanisms of NPs is the generation of reactive oxygen species (ROS), which results in oxidative stress in microbial cells. NPs produce a range of ROS types, including superoxide radicals (O_2), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), by reducing oxygen molecules through redox potential. Oxidative stress plays a crucial role in the destruction of bacterial cells, whereby membrane permeability is compromised. The results of recent researches indicate that oxidative stress leads to DNA damage, apoptosis of bacterial cells, deactivation of proteins, and disruption of enzymatic activities in the periplasm which are crucial for maintaining bacterial morphology and physiological functions (Rout & Pradhan, 2024). In the current study, *in- vitro* antimicrobial tests indicated that the MIC and MBC values of the ZnO-NPs against the tested pathogens were found to be ranged between 20.83 to 41.67, and 66.67 to 83.33 µg/mL, respectively, (Table 1). In addition, the diameter of the inhibition zones exhibited a range of 15.16 to 16.96 mm. The MIC value of the ZnO-NPs against *E. coli* O157:H7 and *S.* Typhimurium were found to be lower than that of *L. monocytogenes*. The lowest MBC value was observed for *E. coli* O157:H7. Besides, inhibition zone of ZnO-NPs against *L. monocytogenes* was found to be larger than that of the other two pathogens. Pauzi et al. (2021), observed that the diameter of the inhibition zone of ZnO-NPs synthesized using gum arabic against *E. coli* (1.4 ± 0.09 cm) was comparable to the findings of our study. Hamk et al. (2023), reported that the diameter of the inhibition zone of ZnO-NPs produced by green synthesis from *Bacillus subtilis* ranged from 12 to 13.3 mm against Gram-negative bacteria. For Gram-positive microorganisms, these values were determined to be 11 to 12 mm. The same researchers documented that the MIC value of the ZnO-NPs against *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium was found to be 2.0, 1.0, and 1.0 mg/mL respectively. In another study, the MIC values of NPs produced from Fennel Seeds Extract were determined as 32.00 µg/mL for *S. aureus*, *S.* Typhimurium, and *Cryptococcus* sp., and 64.00 µg/mL for *P. aeruginosa* (AlSalhi et al., 2020). El-Fallal et al. (2023), investigated the antibacterial effect of ZnO-NPs produced by kombucha extract. They reported that the MIC value exhibited variability againsts tested pathogenic strains, with values of 25, 30, and 40 µg/mL against *E. coli*, *S. aureus*, and *Klebsiella pneumoniae*, respectively. The zone of inhibition was observed to increase in conjunction with an increase in NPs concentration, indicating a positive correlation between the two variables. At a concentration of 50 µg/mL, the zone of inhibition was observed to be 25 for *E. coli*, while at a concentration of 150 µg/mL, the zone of inhibition was 32 mm. The MIC of the ZnO-NPs produced by green synthesis from grape seed extract against *S. aureus* was determined as 62.5 µg/mL in a study conducted by Donmez and Keyvan (2023). Alizadeh-Sani et al. (2020), reported that the MIC value of ZnO-NPs was found to be 2.5 and 3 mg/mL for *E. coli* and *S.* Enteritidis and 2.0 and 1.5 mg/mL for *S. aureus* and *L. monocytogenes,* respectively.

In the present study, the MBC values of ZnO-NPs were found to be approximately two times higher than the MIC value against *L. monocytogenes*, three times for *E. coli* and four times for *S.* Typhimurium. A review of the literature reveals that ZnO-NPs has been demonstrated to possess a potent bactericidal effect by numerous researchers (Arayesh et al., 2023; de Souza et al., 2019; El-Fallal et al., 2023).

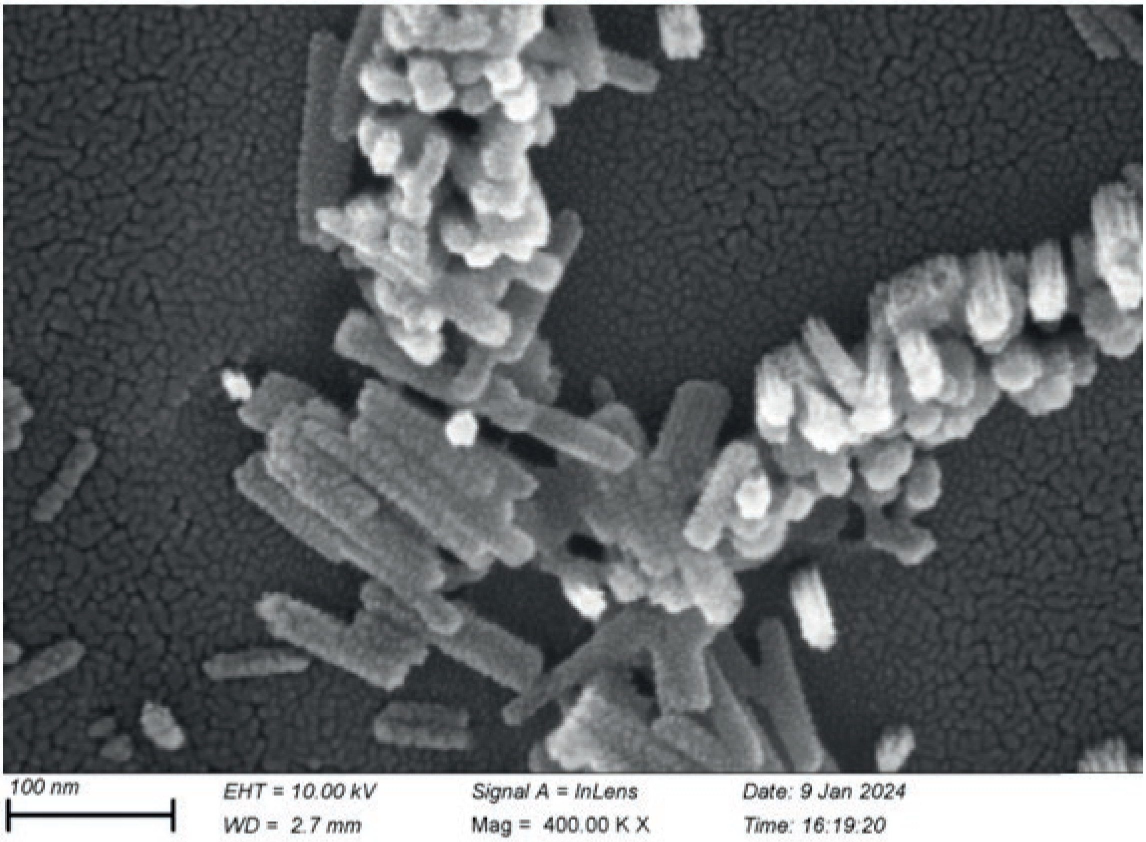

Figure 1. SEM image of the ZnO-NPs produced by hydrothermal method (Magnification was 400.000, and the lenght of bar is 100 nm).

Table 1. The minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and inhibition zone diatemers of ZnO-NPs against some major foodborne pathogens (mean ± SD).

	MIC value (µg/mL)	MBC value (µg/mL)	Inhibition zone (mm)
Listeria monocytogenes ATCC 13932	41.67 ± 14.43	83.33 ± 28.87	16.96 ± 0.61
Escherichia coli O157:H7 ATCC 35150	20.83 ± 7.22	66.67 ± 28.87	15.16 ± 0.32
Salmonella Typhimurium ATCC 14028	20.83 ± 7.72	83.33 ± 28.87	15.77 ± 0.31

ZnO-NPs exhibited a pronounced antimicrobial impact against both Gram-positive and Gram-negative bacteria (Akbar et al., 2019). Nevertheless, the ZnO-NPs used in the current study demonstrated superior antibacterial efficacy against Gram-negative bacteria in comparison to the Gram-positive bacteria. The results of this study align with those of Priyadarshi et al. (2021), who observed a greater antimicrobial activity in *E. coli* compared to *L. monocytogenes*, and Nandhini et al. (2024), who found a higher antimicrobial activity in *E. coli* compared to *S. aureus*. Roy et. al (2021), emphasised that the possible reason for this is due to the difference in the cell wall structure of microorganisms. It is well known that the cell wall of Gram-positive bacteria has a thicker peptidoglycan layer than that of Gram-negative strains, which may limit the penetration of ZnO-NPs into the cell (Hamk et al., 2023). This may explain the higher susceptibility of Gram-negative strains in comparion to the Gram-positive ones against NPs.

The antibacterial activity of metal oxide NPs is influenced by several factors, such as size, surface area to volume ratio, crystalline structure, and surface chemistry. Additionally, the shape of NPs plays a role in their antibacterial effectiveness and mode of action. This shape-related activity is often linked to the interaction between NPs and cell wall surfaces. NPs with a high aspect ratio, having their long axes parallel to the bacterial membrane, exhibit increased surface attachment. Thus, the antibacterial activity of NPs is indirectly related to their surface area. Another important factor is the size of the NPs. NPs with smaller nanoparticles demonstrateg superior antibacterial activity compared to bulk materials, as they can more effectively cover the bacterial surface (Karakaplan, 2021; Ba-Abdad et al., 2017). Differences in the results of the aforementioned studies may be due to variations in production methods, particle size and shape, susceptebility of tested strains, and analysis methods.

On the other hand, potential adverse effects such as safety concerns have been associated with the increasing use of engineered NPs in food industry. The intake of NPS may exhibit a adverse effect on effects gastrointestinal microflora. Furthermore, they absorption of the NPs in gastrointestinal tract may effect different organs and body systems. Threfore, the toxicity, risk assessment, clinical approval, and consumer adaptability of the NPs are still need for further investigation. Further research is required to address these knowledge gaps and to develop a safe and non-toxic NPs for food industry.

Conclusion

The SEM image demonstrated that the nanoparticles had been successfully synthesized by hydrothermal method. It was established that ZnO-NPs with a particle size range of 23 to 25 nm exhibited a pronounced antimicrobial effect against the tested pathogens. It was concluded that the antimicrobial activity of the ZnO-NP was higher in *E. coli* O157:H7 and *S.* Typhimurium compared to *L. monocytogenes* in terms of MIC value. At the MBC value, which represents the dose required for complete microbial eradication, the highest effect (lowest dose) was observed in *E. coli* O157:H7. In terms of zone of inhibition, the most pronounced antibacterial effect was recorded in *L. monocytogenes.* It is thought that NPs produced via the hydrothermal method canbe used as an antibacterial agent to enhance the microbiological quality of foods, due to their pronounced antibacterial properties. However, despite the promising potential of NPs, scalability issues, lack of standard methodologies, and toxicity related to nanoparticle migration also raise concerns. In order to address the current limitations in the widespread adoption of NPs in food preservation technology, detailed interdisciplinary studies in the fields of food science and environmental management are needed to achieve standardized synthesis approaches, characterization of nanoparticle-based additives, safety assessment, and compliance with appropriate labeling requirements and standards for consumer awareness.

Declarations

Ethical Approval

Ethical approval is not required.

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